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# Controlled coculture fermentation for the production of new beverages

**Special strains of *Gluconobacter*, *Lactobacillus* and *Kluyveromyces* have been selected for the production of a new beverage on the basis of wort. This beverage contains the health benefiting substances gluconic acid, lactic acid and an ethanol concentration below 0.5 % (v/v). Fermentation was adapted for growth behaviour and to maximise production – the strategy developed concurrently produces (a) gluconic acid by using the strictly aerobic *Gluconobacter* strain and (b) lactic acid by using the anaerobic strain of *Lactobacillus*. The concentrations of the simultaneously produced organic acids are controlled by the level of oxygen in the fermentation medium. The following mixed fermentation of *Lactobacillus*, *Gluconobacter* and *Kluyveromyces* using the chosen yeast strain resulted in producing a pleasant flavour.**

Descriptors: beverage, *Gluconobacter*, *Kluyveromyces*, *Lactobacillus*, cocultivation

## 1 Introduction

Many microorganisms have long been used for the production of different foods. Traditionally, microbiological fermentation has been used in the production of yoghurt, beer, salami, cheese, sauerkraut and wine. Flavour, agreeableness and storage life can also be increased by using microorganisms. Many foods are produced by cocultivation of different microorganisms. Some examples are sauerkraut, Belgian Lambic beer[1], wine[2], cacao beans[3], cheese[4] and coffee beans[5]. During the fermentation process, growth and product formation of the different microorganisms continually change throughout the different phases of fermentation. The sequence of the microorganisms involved is regulated by conditions such as metabolites, pH, temperature, oxygen availability and substrate concentrations as well as the complex interaction between different microorganisms.

The controlled fermentation of mixed cultures requires a complex fermentation strategy. The influence of changing substrate and product concentrations on the various microorganisms and the parameters already mentioned need to be taken into account[6].

### Bacteria of the genus *Gluconobacter*

Bacteria of the genus *Gluconobacter* are Gram-negative catalase positive rods which belong to the family *Acetobacteraceae*[7]. These strictly aerobic microorganisms are found in fruit and flowers in nature. They are characterized by the incomplete oxidation of carbohydrates, alcohols and related compounds. Glucose is metabolized via the pentose phosphate pathway. Glycolysis is blocked due to a missing phosphofructokinase.

The oxidation of glucose to gluconic acid, which is beneficial for human health, is crucial for the development of the beverage. The oral intake of gluconic acid effects the formation of butyrate in the colon[8] which inhibits cancer formation and may activate apoptosis in cancer cells.

### Bacteria of the genus *Lactobacillus*

*Lactobacillaceae* are Gram-positive facultative anaerobic bacteria. *Lactobacillus L0610*, the strain used, is an obligate homofermentative bacterium. This means that more than 90% of the glucose is metabolized to lactic acid. Lactic acid has a positive influence on colon function and enhances digestibility[9].

Lactic acid bacteria (LAB) are used in the food industry for the production of yoghurt, cheese and sourdough. As L(+)-lactic acid is beneficial to health; strains which form this conformation of lactic acid are preferred. Probiotic strains can be used to increase health benefits in the production of food. These strains are resistant to bile acid, positively influence intestinal flora and improve the immune system[10]. The treatment of colitis[11] and the prevention of cancer[12,13] is discussed with different strains of LAB.

### Yeasts of the genus *Kluyveromyces*

The yeast of the genus *Kluyveromyces* belongs to the family of the *Saccharomycetaceae* and is a Crabtree negative yeast, no ethanol is produced under aerobic conditions, even when glucose concentration is high. *Kluyveromyces* strains are used in the food industry for the production of kefir. Some strains are able to produce flavour compounds such as 2-phenylethanol[14]. Due to high secretory capacity, strains of this yeast are also used for recombinant production of proteins e.g. human serum albumine[15] or human lysozyme[16,4].

### Mixed fermentation

In the field of industrial biotechnology, only one strain is normally selected and cultivated for the manufacture of a particular product under sterile conditions. The presence of other microorganisms has to be avoided. The only exception is waste water treatment and, in some cases, the fermentation of food where mixed cultivations may be utilized.

Most biotransformations in nature take place by the combination of metabolisms from different microorganisms e.g. in large intestine of mammals and in soil. The interaction between the different microorganisms may be advantageous for the strains involved. In other cases there may be competition for substrates and habitat.

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Figures see Appendix

Therefore, microorganisms have developed different strategies to protect substrates and defend their habitat against competitors.

The different requirements of the strains as regards pH value, substrate and product concentrations, optimal temperatures, oxygen availability as well as possible interactions between the strains have to be taken into account for the mixed fermentation process. All the parameters listed may be used to control growth and product formation of the different strains. The literature reports that mixed cultivation of *Lipomyces starkeyi* and *Leuconostoc mesenteroides* producing clinical dextranes[17] is advantageous. Cocultivated *Clostridium thermolacticum* and *Moorella thermoautotrophica* for a thermophilic production of acetic acid[18] and the advantages of ethanol production by a mixed cultivation of *Zymomonas mobilis* and *Saccharomyces sp.* have also been described[19].

#### Formation of flavour substances by microorganisms

A wide range of flavour substances are used for the production of food, cosmetics and drugs. Due to advances in biotechnology, more and more of these substances can be produced by microorganisms. An annual production of several thousand tons[20] of vanillin can be produced via biotransformation using *Pseudomonas putida*[21], *Streptomyces setonii*[22] or *Nocardia sp.*[23]. Another example is the formation of the strawberry ketone, a main component in strawberry flavour[24]. Several yeast strains are able to form different volatiles. Some examples are: *Kluyveromyces lactis*, *Sporobolomyces odorus*, *Geotrichum klebahnii* und *Williopsis saturnus*[24].

This paper describes the production of a fruity beverage on the basis of wort by cocultivation of the three different microorganisms *Kluyveromyces*, *Lactobacillus* and *Gluconobacter*. A method to control the simultaneous formation of L(+)-lactic- and gluconic acid by mixed fermentation of the strictly aerobic acetic acid bacteria and the anaerobic lactic acid bacteria is also presented.

## 2 Material and methods

#### Strains

*Gluconobacter G0104*, *Lactobacillus L0610* and *Kluyveromyces K0304* from the strain collection of the Research Institute for Microbiology, VLB Berlin

#### Medium

The medium used for the experiments in this paper was wort without hops. For wort production, 4 kg crushed pilsmalt (Weyermann Malzfabrik, Bamberg, Germany) were mashed in 25 l of water using the Braumeister (Speidel Tank- und Behälterbau GmbH, Ofterdingen, Germany). Mashing temperature was 62 °C. Temperature was raised to 72 °C after 30 minutes, followed by a further increase to 76 after an additional 30 min. Once all starch was degraded, temperature was increased to 100 °C for at least 30 minutes to reach an original gravity of 12 %. The main carbohydrates were maltose (c = 52 g/l), maltotriose (c = 14 g/l) and glucose (c = 9 g/l). Further parameters were estimated: FAN 189 mg/l; pH = 5.3.

#### Precultures

Precultures were grown in sweet wort at 26 °C for 48 h. Inoculation was done out of a cryo-conserve. Yeast and acetic acid bacteria were grown under aerobic conditions in a volume of 200 ml in 1000 ml Erlenmeyer flasks at 150 rpm, the lactic acid bacteria (LAB) were grown under anaerobic conditions at 26 °C.

#### Bioreactor

Fermentations were carried out in Biostat® Aplus bioreactors (Sartorius Stedim Systems GmbH, Melsungen, Germany) with a maximal working volume of 5 l equipped with heating sleeve and cooling finger, combined with the cooling device Frigomix 1000 (Sartorius Stedim Systems GmbH, Melsungen, Germany), the oxygen probe Oxyferm FDA 325 (Hamilton, Bonaduz, Schweiz) and the pH-probe Easyferm plus K8 325 (Mettler-Toledo GmbH, Gießen, Germany). 20 % (w/v) KOH (Merck KGaA, Darmstadt, Germany) and 20 % (v/v) $H_3PO_4$ (Merck KGaA, Darmstadt, Germany) were applied for pH-control.

#### HPLC

Glucose, maltose, maltotriose, gluconic and lactic acid as well as ethanol were quantified by HPLC-analysis. The HPLC system consisted of the autosampler AS – 96 C, including a column oven at a temperature of 70 °C (Bio-Rad Laboratories, Hercules, USA), a degasser Degasys DG-1310 (Uniflows LTD, Tokyo, Japan), the HPLC Pump 64 (ERC GmbH, Riemerling, Germany) and the column Eurocat H (Knauer GmbH, Berlin, Germany). Sample volume was 20 $\mu$l. The mobile phase was 5 mM $H_2SO_4$ (Merck KGaA, Darmstadt, Germany) at a flow rate of 0.4 ml/min. Detection method: ranged RI- and UV-detectors Smartline 2300 at a temperature of 50 °C and Smartline 2500, at a wave length of 210 nm (Knauer GmbH, Berlin, Germany). Controlling and data processing was done by Chromgate Software, version 3.1 (Knauer GmbH, Berlin, Germany).

## 3 Results and discussion

#### Selection of suitable microorganisms

The objective of the selection process was to produce a beverage containing lactic and gluconic acid, both substances beneficial to health. Hence, special acetic and lactic acid bacteria were selected metabolizing maltose from wort. *L0610*, the lactic acid bacteria chosen forms L (+) lactic acid at a concentration of 15 g/l under anaerobic conditions in the produced wort. The acetic acid bacterium *G0104* produces high amounts of gluconic acid and negligible amounts of acetic acid. Acetic acid is undesirable because of its negative sensorial properties. A low fermentation rate, GRAS (generally recognized as safe) status and the formation of a pleasant flavour were the selection criteria for the used yeast *K0304*. The three different microorganisms were initially incubated in single cultivations to estimate the optimal parameters for product formation.

#### Parameters to control the coculture fermentation

Temperature is an important parameter for influencing growth and production rate of microorganisms and can be used to control coculture fermentation. Hence, the influence of temperature on the acetic acid and lactic acid formation was investigated.

Gluconic acid was produced at a concentration of 55 g/l, whereas maltose concentration was reduced to 21 g/l at the optimal fermentation temperature of 26 °C (Fig. 1). The increase and decrease of temperature resulted in lower concentrations of gluconic acid and a lower degradation of maltose, indicating that the optimal temperature for the production of gluconic acid for the chosen strain is 26 °C. The concentration of glucose was negligible in all incubations after 24 hours.

The concentration of formed lactic acid increased up to 5 g/l lactic acid at 37 °C under anaerobic conditions (Fig.2). The different

temperature optima for *Lactobacillus 0610* and *Gluconobacter 0104* were an opportunity to control product formation during the mixed fermentation process. Increasing temperature up to 37 °C promoted the production of lactic acid, whereas fermentation at lower temperatures increased the formation of gluconic acid to its maximum at 26 °C.

Apart from temperature, the availability of oxygen is also a parameter for controlling growth and product formation of the different microorganisms in mixed fermentation. The acetic acid bacterium *G0104* is a strictly aerobic microorganism, whereas the lactic acid bacterium *L0610* is an aero tolerant organism. It requires anaerobic conditions for optimal growth and metabolism, but will not die in the presence of oxygen. The product formation of the different microorganisms is stimulated by changing temperature and oxygen availability in the different phases of fermentation (Fig. 3). While product formation is enhanced in one strain of the cocultivation fermentation process, one has to ensure that parameters are maintained which do not lead to death of the other strain. Bacterial strains of strictly aerobic gluconic acid bacteria that survive anaerobic conditions had to be selected and oxygen tolerating lactic acid bacteria had to be found. An increase of temperature above 35 °C had to be avoided due to the temperature sensibility of *Gluconobacter ssp.*

The formation of both organic acids by the mixed culture can be achieved by separation of the fermentation process into different phases. During these fermentation processes, the wort produced served as substrate. The pH-value was kept constant at pH = 4.5. Aerobic conditions were achieved by a constant aeration rate of 2 vvm and a stirrer speed of 200 rpm. Phases differ in temperature and aeration or the absence of air (Fig. 3a and b). To enhance lactic acid production in the anaerobic phase, temperature was controlled at 35 °C while gluconic acid production was improved by the optimal temperature of *Gluconobacter G0104* at 26 °C during the aerobic phase. The different phases of fermentation can be interchanged resulting in similar concentrations of 6–9 g/l lactic acid and of 11 g/l gluconic acid. During fermentation of the selected microorganisms, lactic acid is not degraded during the aerobic phase as the selected strain of *Gluconobacter G0104* is unable to oxidise lactic acid and gluconic acid remains stable under anaerobic conditions. The specific taste and flavour of wort were removed during the presented coculture fermentations as also reported by Tenge (2002)[25].

Apart from the differences in temperature optima shown (Fig. 1 and 2), there are also differences in the productivity of the organic acids in relationship to oxygen availability between both strains. The influence of different oxygen transfer rate achieved by headspace or direct aeration and different stirrer speed on the product formation was investigated during mixed fermentation of *Gluconobacter G0104* and *Lactobacillus L0610* (Fig. 4).

In an anaerobic fermentation process, productivity of lactic acid of 0.5 g/(l*h) was observed whereas nearly no gluconic acid was produced. The increase of available oxygen by head space aeration results in a productivity of gluconic acid of 0.1 g/(l*h). The formation of lactic acid decreased to 0.2 g/(l*h). A further increase of available oxygen by direct aeration into the media with an aeration rate of 1 vvm and a stirrer speed of 150 rpm resulted in increased productivity of gluconic acid formation. The highest productivity of 0.58 g/(l*h) of gluconic acid and the lowest productivity of lactic acid of 0.03 g/l(l*h) was observed at the highest oxygen transfer by direct aeration of 2 vvm with a stirrer speed of 200 rpm.

The high oxygen transfer rate is caused by direct aeration and increased stirrer speed. Most of the oxygen is used by the acetic acid bacteria for energy production through the oxidation of glucose to gluconic acid. Biomass formation of *Gluconobacter G0104* is improved by higher aeration rates. The increased oxygen transfer rate shown in Figure 4 inhibits with raising oxygen concentration the formation of lactic acid because of the toxic effect of oxygen onto *Lactobacillus L0610*. Lactobacillus strains are not able to eliminate free oxygen radicals causing damaged proteins, lipids and DNA and resulting in a decreased metabolic activity.

Improving the product formation of one of the members of the coculture decreases product formation conditions for the other member. Because of the fluent change in growth conditions, oxygen availability can be utilized very well to control product formation during the coculture fermentation of *Lactobacillus L0610* and *Gluconobacter G0104*.

A further improvement in the control of product formation shown in Fig. 3 can be achieved by the combination of the oxygen availability with different optimal temperatures of *Gluconobacter G0104* and *Lactobacillus L0610* (Fig. 1 and 2).

Taking into account the requirements of all microorganisms in the mixed culture, the simultaneous production of lactic acid by the anaerobic *Lactobacillus L0610* and the formation of gluconic acid by the strictly aerobic *Gluconobacter G0104* can be achieved (Fig. 5).

Gluconic and lactic acid were produced simultaneously up to 30 g/l within 48 hours, whereas maltose was reduced from 45 g/l to 20 g/l and glucose concentration decreased from 70 g/l to 40 g/l. The typical taste of wort is removed from the fermentation broth after the bacterial production of the organic acids. The production and the end concentration of both organic acids in mixed cultivation can easily be influenced during fermentation by changing the aeration rate. This fact is of great importance when the process is transferred into pilot or production scale because different bioreactor dimensions may cause changed oxygen transfer rates and hence different formation of the organic acids. Furthermore, the production of the organic acids may be adapted to the requirements of different beverages containing different ratios of gluconic and lactic acid.

Inoculation with the yeast *Kluyveromyces K0304* was done subsequent to the production of the organic acids with a concentration of $6*10^6$ cells/ml. Yeast fermentation was performed at 26 °C without aeration and pH adjustment. Several flavour substances were detected by GC-MS analysis of the beverage that contribute to the fruity flavour. The yeast is responsible for the formation of the pleasant flavour and the carbon dioxide production. Fermentation was stopped after 48 h to avoid off-flavour formation and further ethanol production. At the end of fermentation the ethanol concentration was below 0.5 % (v/v).

## 4 Conclusion

A fruity tasting, carbon dioxide containing beverage can be produced on the basis of wort by the described mixed fermentation using the bacteria *Gluconobacter G0104* and *Lactobacillus L0610* in combination with the yeast *Kluyveromyces K0304*. The beverage contains the substances gluconic- and L(+)-lactic acid, which are beneficial to health and can be assessed as "alcohol-free" as the alcohol level is below 0.5 % (v/v).

The strictly aerobic *Gluconobacter G0104* and the anaerobic *Lactobacillus L0610* strains have different optima as regards tem-

perature and oxygen availability. These parameters were selected to control the simultaneous production of the beneficial lactic acid and gluconic acid in mixed cultivation. The concentrations of both organic acids produced can be influenced by changing these two parameters during fermentation, allowing a reproducible quality of the product as well as easy adaptation of the process to other bioreactors.

The development of the reported fermentation strategy gives breweries the opportunity to produce an alcohol-free, fruity product and to reach new consumer markets. Further improvements might be the addition of fruit extracts to increase the antioxidant potential as well as creating beverages with a different taste.

**Abbreviations**

| | | |
|---|---|---|
| v/v | = | volume per volume |
| w/v | = | weight per volume |
| vvm | = | volume per volume and minute |
| rpm | = | rounds per minute |
| FAN | = | free amino-nitrogen |
| LAB | = | lactic acid bacteria |

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## Appendix

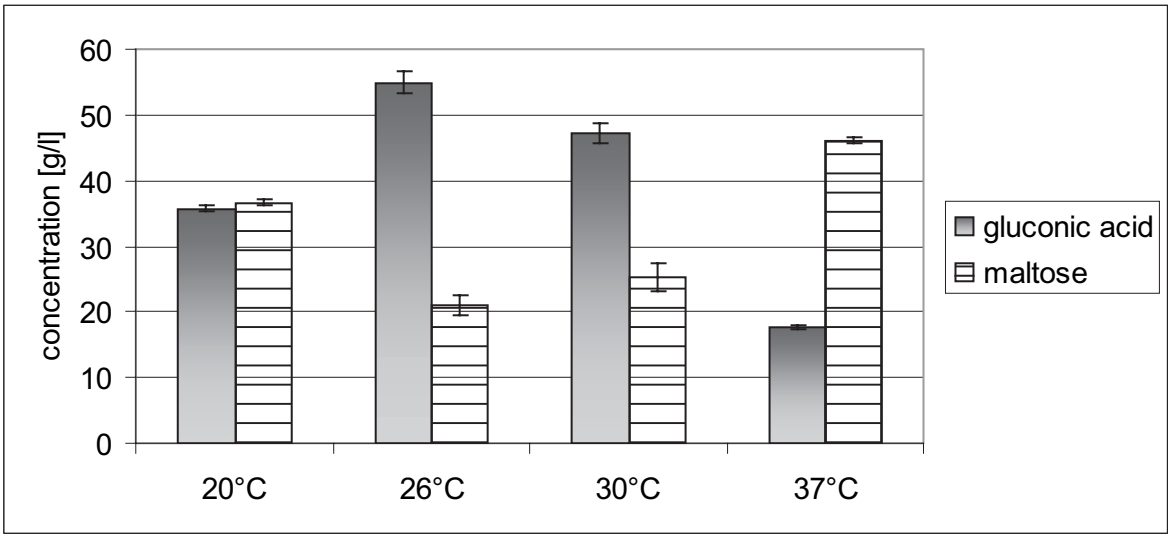


**Fig. 1** **Concentration of the substrate maltose and the gluconic acid formed after 24 hours of aerobic incubation of *Gluconobacter G0104* in shake flasks at different temperatures. Mean values and standard deviation were calculated from 3fold incubation.**

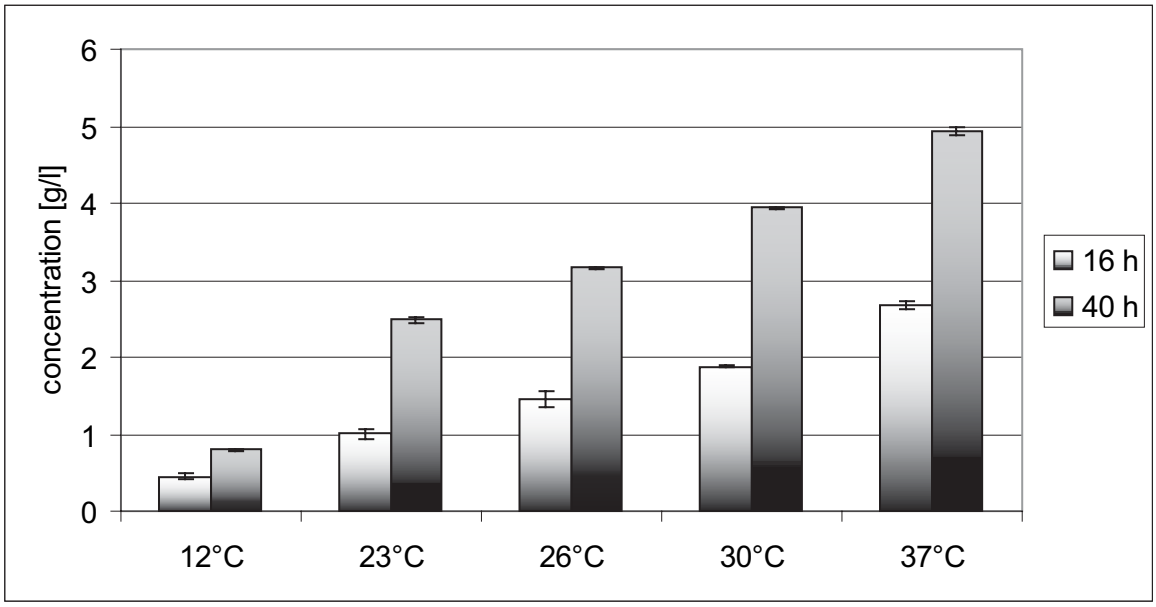


**Fig. 2** **Concentration of lactic acid produced after 16 and 24 hours of anaerobic incubation of *Lactobacillus L0610* at different temperatures. Mean values and standard deviation were calculated from 5fold incubation.**

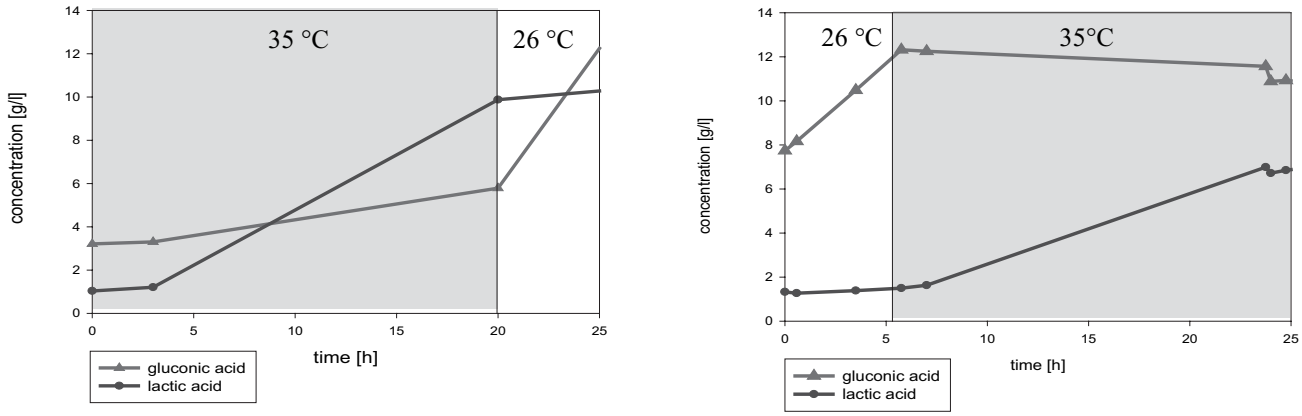


**Fig. 3** **Product formation of divided mixed fermentation of *Gluconobacter G0104* and *Lactobacillus L0610* a) (left): anaerobic phase (grey) followed by aerobic phase (white); b (right) aerobic phase (white) followed by anaerobic phase (grey)**

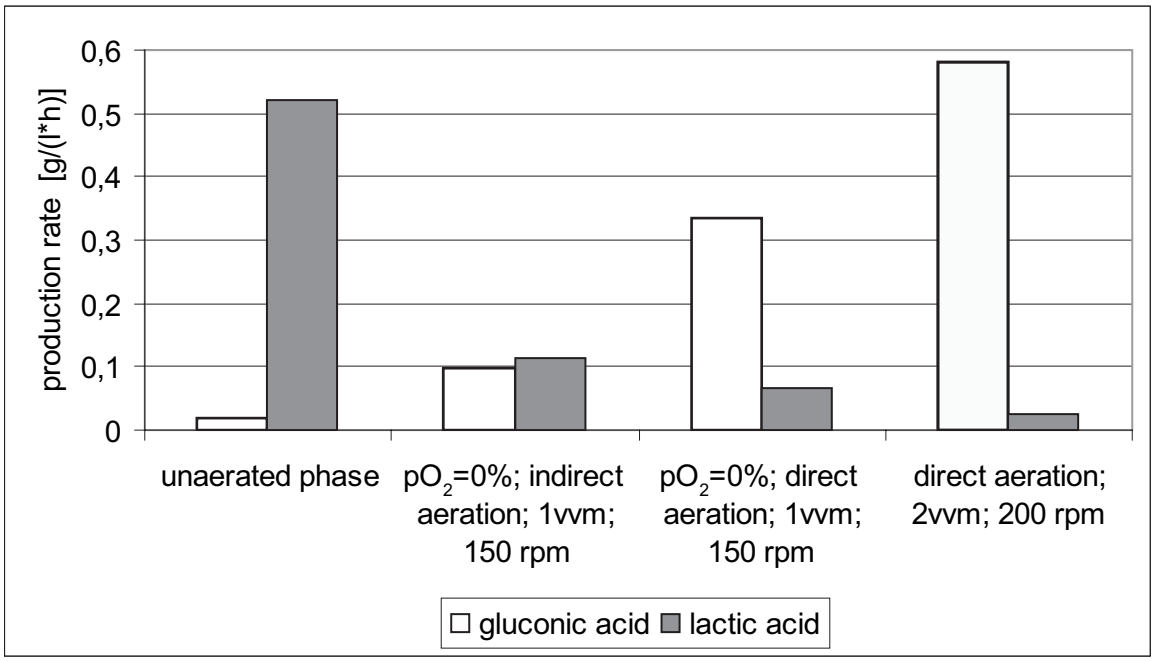


**Fig. 4** **Influence of aeration on the productivity of gluconic and lactic acid by the mixed culture of *Gluconobacter G0104* and *Lactobacillus L0610***

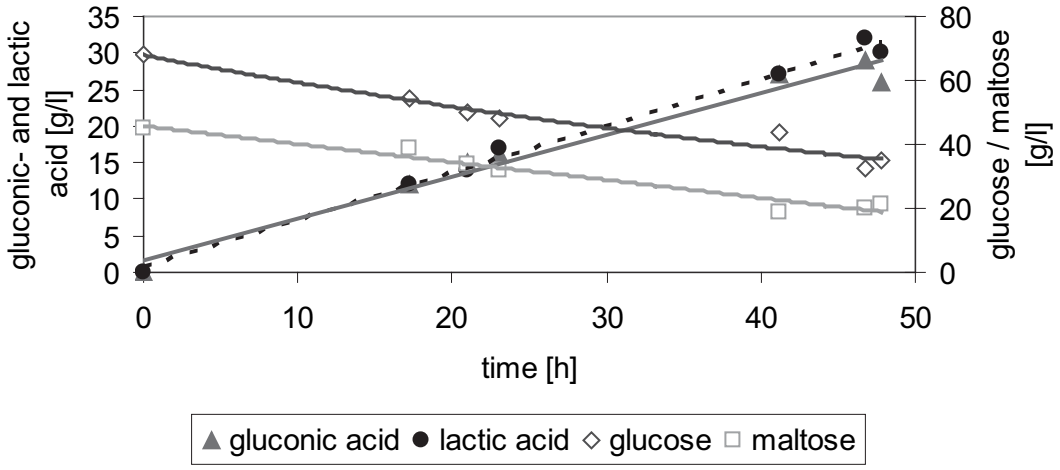


**Fig. 5** **Gluconic and lactic acid, maltose and glucose concentrations during the mixed fermentation of *Gluconobacter G0104* and *Lactobacillus L0610***